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Two Distinct Mechanisms Target Membrane Proteins to the Axonal Surface

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Summary

We have investigated the trafficking of two endogenous axonal membrane proteins, VAMP2 and NgCAM, in order to elucidate the cellular events that underlie their polarization. We found that VAMP2 is delivered to the surface of both axons and dendrites, but preferentially endocytosed from the dendritic membrane. A mutation in the cytoplasmic domain of VAMP2 that inhibits endocytosis abolished its axonal polarization. In contrast, the targeting of NgCAM depends on sequences in its ectodomain, which mediate its sorting into carriers that preferentially deliver their cargo proteins to the axonal membrane. These observations show that neurons use two distinct mechanisms to polarize proteins to the axonal domain: selective retention in the case of VAMP2, selective delivery in the case of NgCAM.

Introduction

Nearly all neurons are polarized into two structurally and functionally distinct domains, the axon and the dendrites. Consistent with the different physiological properties of axons and dendrites and their different roles in cell signaling, many cell surface proteins are preferentially distributed either to the axonal or somatodendritic domain. In a general sense, the trafficking pathways involved in the biosynthesis of integral membrane proteins are well understood. These proteins are synthesized in the rough endoplasmic reticulum, pass through the Golgi complex, and are packaged into carrier vesicles, which are transported into the axons and dendrites where they deliver their contents to the plasma membrane by exocytic fusion (Craig and Banker, 1994). With respect to the trafficking of proteins destined for different destinations within the cell, however, many fundamental questions remain unanswered. Where along these pathways does the trafficking of axonal and dendritic proteins diverge? What underlying mechanisms lead to the selective localization of such proteins on the cell surface? With regard to the second question, there are two general mechanisms that could account for the selective localization of polarized proteins on the cell surface. Selectivity along the trafficking pathways en route to the plasma membrane could ensure that proteins destined for different domains are segregated from one another into different carriers and only delivered to the plasma membrane of the appropriate domain. Alternatively, proteins could be delivered equally to the plasma membrane of both domains but retained on the cell surface only in the appropriate domain.

Both mechanisms, selective delivery and selective retention, contribute to the maintenance of polarity in epithelial cells (Matter, 2000). Many apical proteins and basolateral proteins are sorted into distinct transport carriers, either as they exit the Golgi complex or within an endosomal compartment in the cell periphery, and these carriers deliver their cargoes exclusively to the apical or basolateral surface. Other proteins, such as Na,K-ATPase (Hammerton et al., 1991) and β1 integrin (Gut et al., 1998), are delivered in equal amounts to both domains. The proteins that reach the inappropriate domain are rapidly removed by endocytosis, whereas those that reach the appropriate domain interact with submembranous cytoskeletal proteins, which stabilize them in the membrane and prevent their endocytosis.

Studies of neuronal protein targeting have revealed motifs that are required for their appropriate localization (West et al., 1997b; Jareb and Banker, 1998; Arnold and Clapham, 1999; Stowell and Craig, 1999; Lim et al., 2000; Ruberti and Dotti, 2000; El-Husseini et al., 2001; Eiraku et al., 2002), but it is unclear if these motifs mediate selective sorting and delivery or selective retention. Both mechanisms could plausibly contribute to the maintenance of polarity in nerve cells, and there have been few experimental tests to distinguish between them. In the case of dendritic targeting, the available evidence favors the selective sorting and delivery model. The same motifs that govern the targeting of some dendritic proteins in neurons have been shown to mediate selective delivery to the basolateral surface in epithelial cells (Matter and Mellman, 1994; West et al., 1997b; Jareb and Banker, 1998; Nelson and Yeaman, 2001). Moreover, live-cell imaging studies have shown that carriers labeled by expression of GFP-tagged transferrin receptor, a dendritic protein, are transported into dendrites but excluded from axons, implying that such carriers deliver their protein contents only to the somatodendritic membrane (Burack et al., 2000). Similar results have been observed for several other GFP-tagged dendritic proteins, including the acidic amino acid transporter EAAT3, the EGF receptor, and the metabotropic glutamate receptor mGluR1a (Cheng et al. (2002); S. Das, M. Silverman, and G.B., unpublished data), indicating that selective sorting and delivery is likely to contribute to the polarization of many dendritic proteins.

The mechanisms that underlie the polarization of axonal proteins are less well understood, but several lines of evidence raise the possibility that selective retention rather than selective delivery may play a particularly important role. First, in contrast to the situation for dendritic proteins, transport carriers labeled following expression of the GFP-tagged axonal protein NgCAM are

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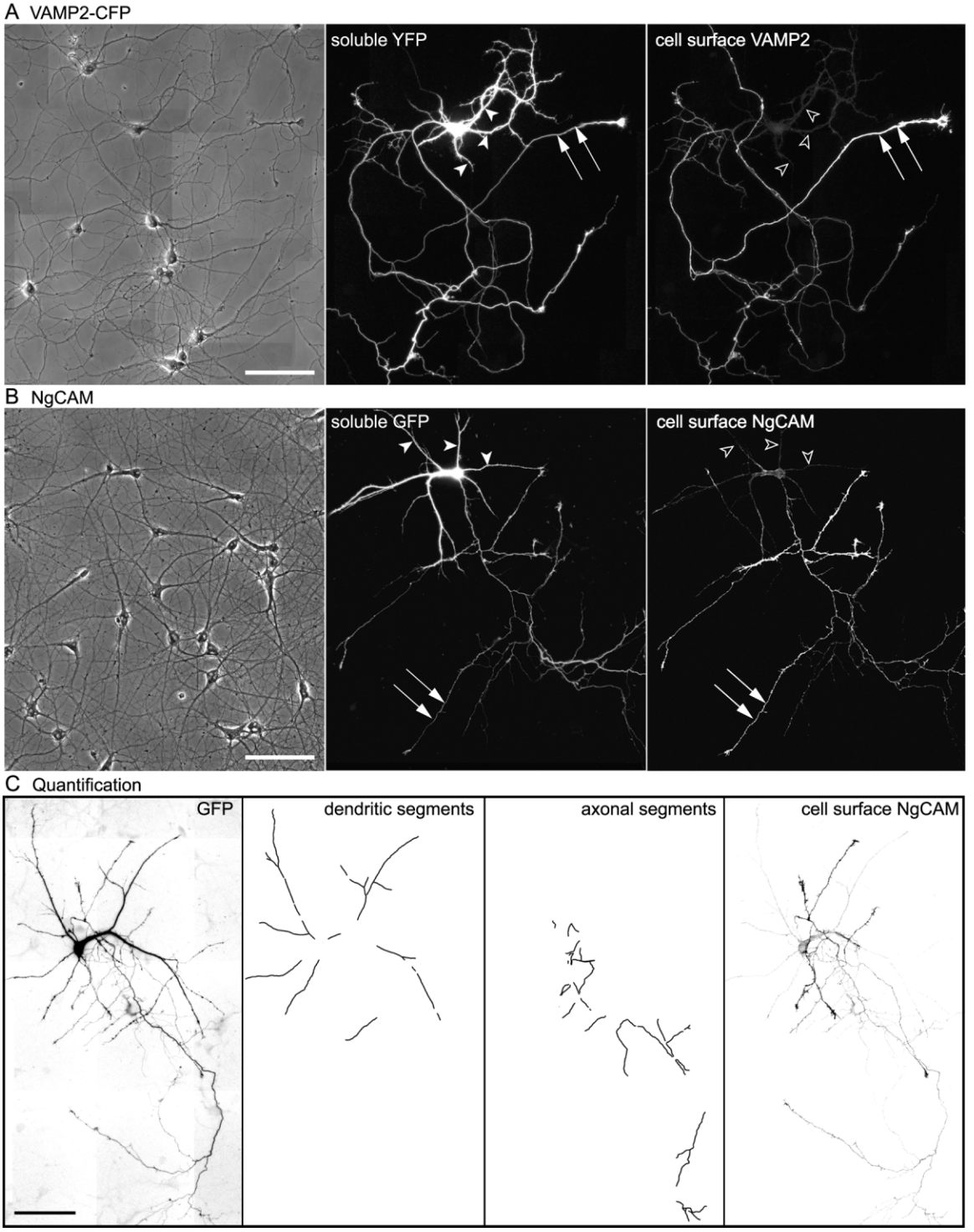


Figure 1. VAMP2 and NgCAM Are Polarized to the Axonal Surface

(A) Transfected VAMP2-CFP was present all along the surface of the axon (arrows), but was conspicuously absent from the dendritic surface (open arrowheads). Primary hippocampal neurons from E18 rat embryos were cultured for 8–11 days in vitro prior to cotransfection of plasmids encoding soluble YFP and CFP-tagged VAMP2. YFP fluorescence (middle panel) reveals the morphology of the transfected cell within a network of untransfected neurons and their processes visible in the phase contrast micrograph (left panel). YFP filled the entire length of the single, branched axon (arrows) and all dendrites (arrowheads). Cell surface localization of the transfected VAMP2-CFP was determined by live immunolabeling using antibodies against the extracellular CFP tag (right panel). Scale bar, 100 μm.

(B) Transfected NgCAM targets to the axonal cell surface. NgCAM was coexpressed with soluble GFP (middle panel). Live immunolabeling with antibody (monoclonal 8D9) against the extracellular domain of NgCAM revealed robust staining along some (arrow), but not all branches of the axon. None of the dendrites showed significant NgCAM at the cell surface (open arrowheads). Scale bar, 100 μm.

transported into dendrites as well as axons (Burack et al., 2000). It is not known if the NgCAM-containing carriers that enter dendrites fuse with and deliver their contents to the dendritic membrane or simply are returned to the cell body and eventually reach the axon. Second, axons and dendrites differ in the molecular composition of their submembranous cytoskeleton (Kunimoto, 1995) and in the components of their endocytic machinery (Sheehan et al., 1996; Wilson et al., 2000; Steiner et al., 2002), which could allow for the selective retention of proteins in the axon. In fact, Garrido et al. (2001) have shown that a construct consisting of the cytoplasmic tail of an axonal sodium channel ($Na_V1.2$) fused to the transmembrane and extracellular domain of a reporter protein (CD4-$Na_V1.2$Cyt) is polarized to the axonal surface by selective retention. CD4-$Na_V1.2$Cyt is endocytosed from the dendritic surface, indicating that the chimeric protein is delivered to both the axonal and dendritic membrane. In addition, mutation of an endocytosis signal present in the cytoplasmic domain of this construct disrupts the polarization of the resultant protein. Despite such suggestive evidence, it is not known if selective retention accounts for the polarization of the full-length $Na_V1.2$ channel, or any other axonal protein.

In this report, we analyze the trafficking of two endogenously expressed axonal proteins, NgCAM and VAMP2, in order to determine which mechanism, selective retention or selective delivery, accounts for their polarity. L1 and its chick homolog NgCAM are members of the Ig superfamily of neural cell adhesion molecules, which are thought to play a role in axonal pathfinding and fasciculation (Hortsch, 1996; Sonderegger, 1998; Demyanenko et al., 1999). When NgCAM is expressed in cultured hippocampal neurons, it is highly polarized to the axonal surface (Jareb and Banker, 1998; Silverman et al., 2001). VAMP2 is a synaptic vesicle v-SNARE that is required for calcium-dependent exocytosis at presynaptic specializations (Jahn and Sudhof, 1999; Schoch et al., 2001). Although VAMP2 is a component of synaptic vesicles, a significant fraction of VAMP2 is also present on the axonal surface (Taubenblatt et al., 1999; Ahmari et al., 2000; Sankaranarayanan and Ryan, 2000). GFP-tagged VAMP2 is highly polarized to the axonal surface when expressed in cultured hippocampal neurons (see below), and carriers containing GFP-tagged VAMP2 are transported into both dendrites and axons (B.S. and G.B., unpublished data), like those containing NgCAM (Burack et al., 2000). Thus, existing data concerning the trafficking of both NgCAM and VAMP2 are equally compatible with the selective retention and selective delivery models. In order to determine which mechanism accounts for the polarization of these proteins, we have assessed whether these proteins are preferentially endocytosed from the dendritic surface. We have also identified regions within these proteins that are required for their polarization to the axonal surface and investigated whether these regions are likely to mediate selective retention or selective sorting. Our findings indicate that the polarization of these two axonal proteins depends on distinct mechanisms: selective retention in the case of VAMP2, selective delivery in the case of NgCAM.

Results

VAMP2 and NgCAM Are Both Polarized to the Axonal Cell Surface

To assess the distribution of VAMP2 and NgCAM, we transfected these constructs into cultured hippocampal neurons and determined their localization after 20–24 hr (Figure 1). In order to visualize the morphology of the cells expressing the constructs, we coexpressed soluble GFP or one of its color variants, which diffuses throughout the cell, labeling all branches of the axon and dendrites. This helps interpretation since staining intrinsic to the dendrites can be difficult to distinguish from staining of axons that fasciculate with the dendrites. Cell surface protein was detected by live-cell immunostaining. NgCAM was detected with species-specific antibodies. Because no antibodies are available against the short extracellular domain of VAMP2 and because the protein is expressed endogenously by hippocampal neurons, we added a CFP tag at the end of its extracellular domain (Ahmari et al., 2000; Sankaranarayanan and Ryan, 2000). Cell surface VAMP2 was detected with antibodies recognizing the CFP tag.

When expressed in hippocampal neurons, both proteins were highly polarized to the axonal surface (Figure 1). Dendrites, which were brightly labeled with soluble YFP, were effectively unstained with antibodies that detect expressed VAMP2 or NgCAM. The details of axonal staining differed somewhat for the two proteins. Staining for VAMP2 was uniformly distributed along the axon, varying in intensity largely as a function of axonal diameter (as revealed by the YFP fluorescence). In contrast, NgCAM staining was brighter in distal axons, consistent with evidence that NgCAM is preferentially inserted in the distal axon (Vogt et al., 1996). Some axonal branches, which were clearly visible by YFP fluorescence, expressed little or no NgCAM.

The following procedure was developed to provide an unbiased assay of the polarization of these axonal proteins (Figure 1C). Lines were traced along the axons and dendrites in the images illustrating soluble GFP. This allowed us to exclude regions where axons and

(C) Quantification of the cell surface distribution of a protein of interest, in this case NgCAM, based on coexpression of soluble GFP. Based on the signal of the cotransfected GFP, which fills the entire cell, an array of images was acquired to cover the entire dendritic and axonal arbor of the transfected neuron. Simultaneously, corresponding images of the cell surface labeling were captured. Using the soluble marker protein as a guide (left panel, inverted contrast), 1 pixel-wide lines were laid along all stretches of nonfasciculated dendrites (second panel) and representative, nonfasciculated segments extending throughout the entire axonal arbor (third panel). Traced lines were transferred in register to the images of the cell surface staining, and the average intensity above background was determined independently for all dendritic and axonal segments. To normalize measurements across cells with varying expression levels, ratios of average axonal versus dendritic staining were calculated for each individual cell; the axon:dendrite ratio of the illustrated NgCAM-transfected cell was 4.0. Choosing segments for analysis based on the signal of a soluble marker protein ensured unbiased sampling, including regions of the axonal and dendritic arbor where cell surface expression of the test protein was not detectable. Scale bar, 100 μm.

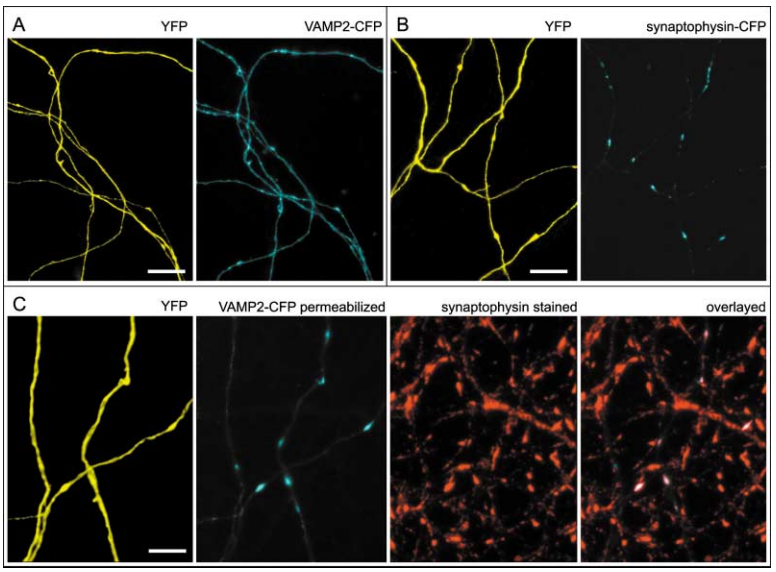


Figure 2. VAMP2 Is Present Both Synaptically and Nonsynaptically in Axons

(A) In nonpermeabilized cells, VAMP2-CFP fluorescence is detected all along the axonal cell surface. High-magnification images of axonal branches of a 10-day-old neuron coexpressing soluble YFP (left panel) and CFP-tagged VAMP2 (right panel). VAMP2-CFP fluorescence was observed all along the axon, with slight enrichment at intermittent varicosities. Scale bar, 10 μm.

(B) Synaptophysin-CFP is restricted to synaptic varicosities. In contrast to the ubiquitous distribution of VAMP2-CFP, synaptophysin-CFP (right panel) was found in puncta along axons (identified by YFP signal, left panel). Scale bar, 10 μm.

(C) VAMP2-CFP localizes mainly to synaptic terminals in cells that have been fixed and detergent extracted. VAMP2-CFP (second panel) is highly concentrated in puncta along the axon (revealed by soluble YFP, left panel). The punctae of VAMP2-CFP correspond to presynaptic specializations, identified by immunolabeling of endogenous synaptophysin (third panel). Colocalization of VAMP2 (cyan) and synaptophysin (red) is best appreciated in the overlay (right panel; colabeling with both fluorophores appears white). Scale bar, 5 μm.

dendrites fasciculated with one another and to sample portions of the axon and dendrites that lacked the expressed protein. The lines were then transferred in register to images of the live-cell immunolabeling, and the fluorescence along these lines was measured and averaged for the dendrites and axon. Using this method, the ratio of axonal to dendritic fluorescence was 6.4 ± 1.1 for VAMP2 and 4.1 ± 0.7 for NgCAM. The lower axon:dendrite ratio for NgCAM reflects the fact that some axonal branches lack NgCAM staining. For comparison, unpolarized proteins exhibit an axon:dendrite ratio between 0.5 and 1.0 (see below). (A priori, one might expect unpolarized proteins to exhibit a 1:1 ratio of dendritic to axonal staining, since only cell surface protein is assessed and since the light path passes through the surface membrane twice [upper and lower surfaces of the neurites], independent of neurite diameter. In practice, staining often appears somewhat brighter in the dendrites than the axon. We believe this reflects the contribution of out-of-focus fluorescence, which is greater in dendrites because of their larger diameter.)

The presence of expressed VAMP2 along the surface of the axon was not a consequence of mistargeting due to overexpression or addition of CFP to its extracellular domain. Instead, it reflects the intrinsic properties of this protein. Unlike other synaptic vesicle proteins, a significant fraction of both endogenous and expressed VAMP2 is found on the plasma membrane (Taubenblatt et al., 1999; Sankaranarayanan and Ryan, 2000). To determine whether expressed VAMP2 also was associated with synaptic vesicles under the conditions used in our experiments, we imaged VAMP2 based on its CFP tag, which demonstrates intracellular as well as cell surface protein. Consistent with the results of Sankaranarayanan and Ryan (2000), fluorescence was present along the axon but concentrated at synaptic varicosities (Figure 2A). In contrast, synaptophysin-CFP, another synaptic vesicle protein, expressed in sister cultures was restricted to synaptic specializations (Figure 2B). Hannah et al. (1998) have reported that fixation and Triton X-100 permeabilization, as commonly employed for immunostaining synaptic vesicle proteins, results in the selective extraction of endogenous VAMP2. When hippocampal cultures expressing VAMP2-CFP were similarly extracted, much of the cell surface immunofluorescence was lost, causing VAMP2-CFP fluorescence to appear restricted to presynaptic specializations, where it colocalized with endogenous synaptophysin (Figure 2C).

VAMP2 but Not NgCAM Is Endocytosed in Dendrites

Dargent and colleagues have proposed that the axonal polarization of neuronal proteins depends on selective retention, not selective delivery (Garrido et al., 2001). They have found that an axonally polarized sodium channel chimeric protein is rapidly endocytosed from the dendritic surface, whereas it is retained and accumulates in the axonal membrane. Both NgCAM and VAMP2 have well-defined endocytosis signals in their cytoplasmic domains. If expressed VAMP2 and NgCAM are continuously added to the dendritic surface but rapidly endocytosed, it should be possible to label endosomes by prolonged incubation of living cells with antibodies directed against extracellular epitopes of these proteins, even though steady-state levels of cell surface protein are low. We exposed cells to primary antibodies for 20 min at 37°C, then fixed them and added fluorescently labeled secondary antibodies either with or without permeabilization. The latter condition only reveals proteins present on the cell surface at the time of fixation (Figure 3A, left panel), while the former method also detects proteins that have been endocytosed from the cell surface (right panel). Under these conditions, VAMP2-

expressing cells that were permeabilized following exposure to antibody exhibited many highly fluorescent puncta within their dendrites. These fluorescent puncta partially colocalized with early endosomes labeled by expression of CFP-rab5 (see Supplementary Figure S1 at http://www.neuron.org/cgi/content/full/37/4/611/DC1). Some anti-VAMP2 was also associated with recycling endosomes, labeled by expression of rab11 (data not shown). No puncta were seen when cells were labeled without permeabilization, confirming that these structures are intracellular and correspond to VAMP2-containing endosomes. These results suggest that VAMP2 is endocytosed from the dendritic surface. Bright staining of VAMP2 in the axonal membrane prevented us from visualizing endocytosis of VAMP2 in the axon (and hence from comparing it with endocytosis of VAMP2 in dendrites), but other methods have demonstrated that VAMP2 is endocytosed from the plasma membrane at presynaptic specializations (Sankaranarayanan and Ryan, 2000; Li and Murthy, 2001). It is possible that some VAMP2 endocytosed from the axonal surface could reach the dendrites via retrograde transport within the 20 min period of our assay, contributing to the VAMP2-labeled endosomes we observed in dendrites (Prekeris et al., 1999).

We obtained very different results when we used an identical assay to assess dendritic endocytosis of NgCAM. In the majority of cells, we were unable to detect any endocytosis of NgCAM, even when the time of incubation was extended to 40 min. As illustrated in Figure 3C, staining with anti-NgCAM was restricted to the axon, whether or not the cells were permeabilized. In a subpopulation of cells, brightly labeled endocytic vesicles were detected in dendrites following incubation with anti-NgCAM and permeabilization (data not shown). Compared with VAMP2-containing endosomes, those labeled with anti-NgCAM were larger and fewer in number. The few neurons that exhibited endocytosis of NgCAM had an immature morphology, characterized by a shorter axon and thinner, less-branched dendrites (as visualized by cotransfected, soluble GFP). In all cells (both the small population that exhibited endocytosis and the majority that lacked endocytosis), cell surface NgCAM was restricted to the axon. These observations indicate that dendritic endocytosis of NgCAM is not essential for its axonal polarization in mature hippocampal neurons.

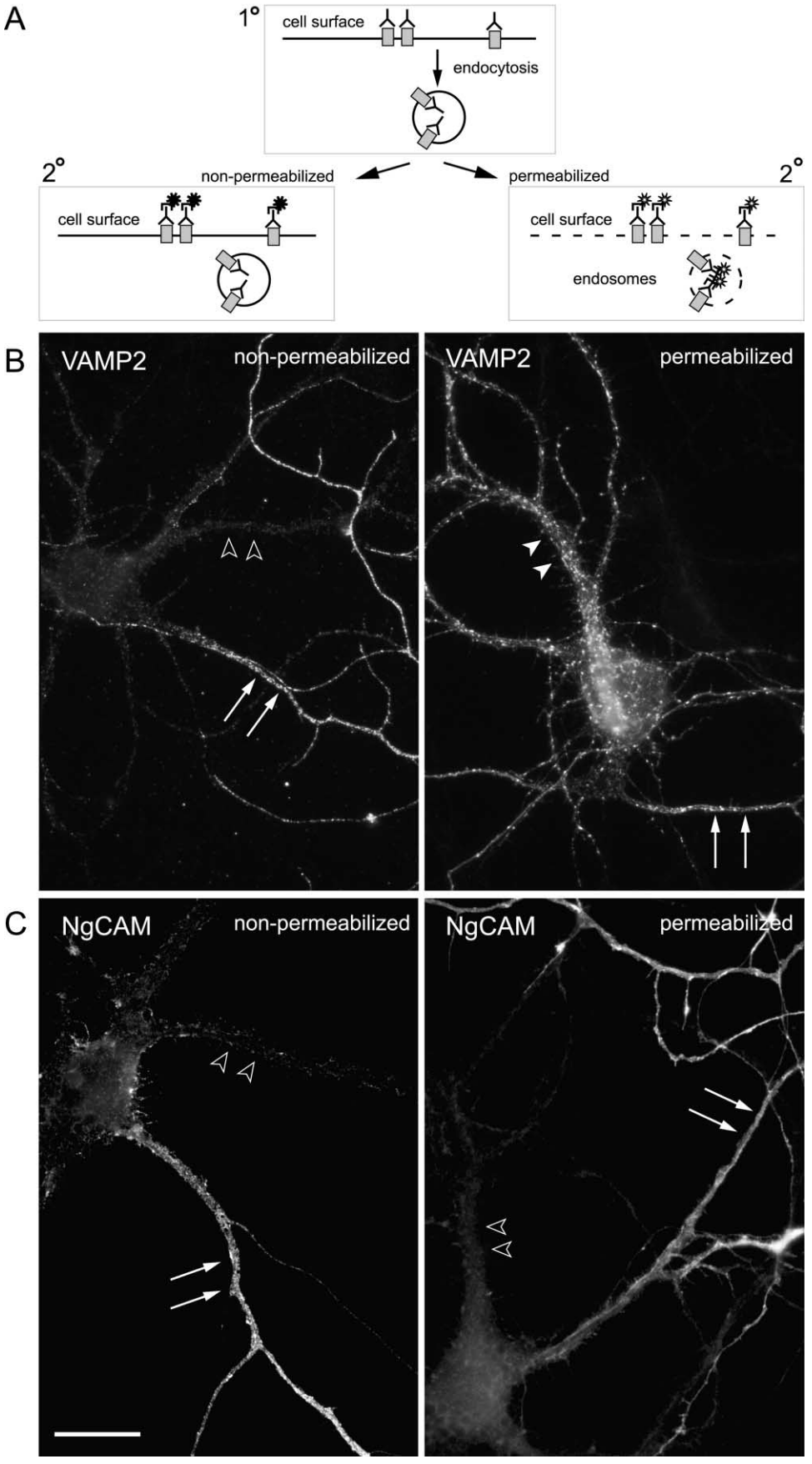
Inactivation of Cytoplasmic Motifs Required for Endocytosis Disrupts the Polarization of VAMP2 but Not NgCAM

A second prediction of the Dargent model is that blocking endocytosis will disrupt the polarization of the axonal proteins. We therefore determined whether inactivation of the endocytosis signals of VAMP2 and NgCAM resulted in the appearance of these proteins on the dendritic surface.

In the case of VAMP2, it has been shown that mutation of a single amino acid (M46A) prevents its endocytosis (Grote and Kelly, 1996). When this mutant construct was expressed in hippocampal neurons, its cell surface distribution was markedly different from that of wild-type VAMP2 (compare Figure 4A with Figure 1A). The cell body and dendrites were brightly labeled, with labeling extending even to the finest dendritic branches. Quantitative analysis revealed that the axon:dendrite ratio for the mutant construct was 1.0 ± 0.4, equivalent to values for unpolarized proteins. When living cells expressing the mutant construct were exposed to antibody for prolonged periods, then fixed and permeabilized, no endocytic vesicles were detected in dendrites (see Supplementary Figure S2 at http://www.neuron.org/cgi/content/full/37/4/611/DC1). These results demonstrate that the endocytosis signal in the cytoplasmic domain of VAMP2 is required for its polarization to the axonal surface.

NgCAM contains a canonical AP2 recognition motif (YRSL) within its cytoplasmic tail, and mutation of this site in L1, the mammalian homolog of NgCAM, blocks its endocytosis (Kamiguchi et al., 1998). To assay the significance of this endocytosis motif for the axonal polarization of NgCAM, we prepared several constructs whose cytoplasmic domain had been altered to disrupt its endocytosis (Figure 4C) and assessed their localization following expression in hippocampal neurons. In one case we replaced the transmembrane and cytoplasmic domains of NgCAM with those of CD8α, a lymphocyte protein that lacks known endocytosis signals and that is unpolarized when expressed in hippocampal neurons (Jareb and Banker, 1998). Based on live-cell staining, NgCAM-CD8α was present on the axonal surface but was barely detectable on the dendritic surface. When such cells were incubated with antibodies directed to the extracellular domain of NgCAM for prolonged periods, we were unable to detect endocytic vesicles in the dendrites of any cells, even in cells that appeared less mature based on their morphology (data not shown). Quantitative analysis revealed that this construct was as polarized as wild-type NgCAM (Figure 4C). Similar results were obtained when the transmembrane and cytoplasmic domains were replaced with a GPI anchor. A mutant NgCAM protein lacking the entire cytoplasmic domain also remained highly polarized, although its axon:dendrite ratio was slightly reduced compared with the wild-type protein (Figure 4C). One difference was noted between the distribution of these three constructs and the wild-type protein. Whereas wild-type NgCAM was often concentrated in the distal axon, NgCAM mutants lacking the cytoplasmic domain were uniformly distributed along the axon (compare Figure 4B with Figure 1B). This suggests that maintaining the concentration of NgCAM near its site of addition at the growth cone depends on protein:protein interactions mediated via its cytoplasmic tail. A chimera consisting of the cytoplasmic tail and transmembrane domain of NgCAM fused to the ectodomain of CD8α was completely unpolarized, indicating that the cytoplasmic tail cannot redirect unpolarized proteins to the axon.

In sensory ganglion neurons, it has been reported that inactivation of the endocytosis signal in L1 prevents the protein from reaching the axonal membrane (Kamiguchi et al., 1998). To determine whether the endocytosis motif played a role in the trafficking of L1 in cultured hippocampal neurons, we assessed the polarization of full-length human L1 and of an L1 construct truncated just proximal to the endocytosis signal. Both constructs reached the cell surface and were highly polarized to


A
1°
cell surface
endocytosis
2°
non-permeabilized
cell surface
permeabilized
2°
cell surface
endosomes
B
VAMP2
non-permeabilized
VAMP2
permeabilized
C
NgCAM
non-permeabilized
NgCAM
permeabilized

the axon (data not shown). Thus, as for NgCAM, endocytosis of L1 is not required for establishing the protein's polarized distribution in hippocampal neurons.

Taken together, these results further illustrate differences in the mechanisms underlying the polarization of VAMP2 and NgCAM. The axonal polarization of VAMP2 depends on endocytosis, which is mediated by a signal in its cytoplasmic domain. In contrast, the cytoplasmic domain of NgCAM, which also contains an endocytosis signal, is unimportant for the polarization of NgCAM. This implies that the axonal polarization of NgCAM depends on signals present in its ectodomain.

Sequences in the Ectodomain of NgCAM Are Necessary and Sufficient for Axonal Targeting

The ectodomain of NgCAM is composed of two parts, a membrane-distal domain consisting of six immunoglobulin-like (Ig) repeats and a membrane-proximal domain consisting of five fibronectin type III-like (FnIII) repeats. To determine which of these regions is important for axonal targeting, we deleted each separately. The results of this experiment are illustrated in Figure 5. Following deletion of the six Ig repeats, NgCAM remained highly polarized, although its axon:dendrite ratio was slightly reduced compared to wild-type (2.4 $\pm$ 0.2 versus 3.6 $\pm$ 0.6). In contrast, deletion of the FnIII repeats completely abolished axonal polarization. The axon:dendrite ratio of this mutant was 0.6 $\pm$ 0.1, identical to that observed with unpolarized proteins such as CD8α (axon:dendrite ratio 0.6 $\pm$ 0.1). These results show that the FnIII repeats are necessary for the polarization of NgCAM. Deletions of individual FnIII domains or pairs of FnIII domains reduced axonal polarization slightly (Figure 5D). The greatest reduction was observed following deletion of domains Fn2 and Fn3 (axon:dendrite ratio of 2.0 $\pm$ 0.3). Nonetheless, this construct was clearly polarized compared to the mutant lacking all FnIII domains.

Previous studies have suggested that *N*-glycosylation may act as an axonal sorting signal (Tienari et al., 1996). Because NgCAM contains multiple glycosylation sites within its FnIII domains, we assessed the effect of inhibiting *N*-glycosylation on its polarization. Treatment with tunicamycin (5 μg/ml) for 18 hr did not alter the polarization of expressed NgCAM (axon:dendrite ratio 3.9 $\pm$ 0.3), although the level of its cell surface expression was significantly reduced.

Although endocytosis is usually mediated by signals in the cytoplasmic domain, deletion of the FnIII repeats in NgCAM could have impaired endocytosis of the mutant protein, and this might have contributed to its loss of polarization. To exclude this possibility, we assayed the endocytosis of NgCAMΔFn1-5 by antibody uptake using the method described in Figure 3A. Large, brightly labeled puncta corresponding to endocytic vesicles were readily detected in the dendrites of cells expressing NgCAMΔFn1-5 (Figure 5C). These results demonstrate that dendrites contain endocytosis machinery capable of removing NgCAM from the cell surface, but that this endocytosis does not prevent this construct from accumulating on the dendritic surface.

Finally, we examined the localization of NrCAM, another member of the L1 family of cell adhesion molecules with the same topological organization as NgCAM (Grumet et al., 1991). When expressed in hippocampal neurons, NrCAM was unpolarized (axon:dendrite ratio 0.7 $\pm$ 0.1; see Figure 5D). To determine whether the FnIII domains of NgCAM contain axonal targeting information, we prepared a construct whose ectodomain consisted of the Ig repeats of NrCAM and the FnIII domains of NgCAM. The chimeric protein was selectively targeted to the axon with efficiency similar to wild-type NgCAM. These results demonstrate that the FnIII domains of NgCAM contain sufficient information to redirect an unpolarized protein to the axon.

Discussion

We have investigated the trafficking of two endogenous axonal membrane proteins, VAMP2 and NgCAM, in order to elucidate the cellular events that underlie their polarization. We found that VAMP2 is delivered equally to the surface of both axons and dendrites, but preferentially endocytosed from the dendritic membrane. In contrast, the targeting of NgCAM depends on sequences in its ectodomain, which mediate its sorting into carriers that preferentially deliver their cargo proteins to the axonal membrane. These observations show that neurons use two distinct mechanisms—selective delivery and selective retention—to polarize proteins to the axonal membrane.

Selective Endocytosis Is Responsible for the Axonal Polarization of VAMP2

Our results show that the polarization of VAMP2 is mediated by selective retention, not by selective delivery. We used antibody labeling of living cells to assay the amount of VAMP2 present on the cell surface (by addi-

Figure 3. Endocytosis of VAMP2 but Not NgCAM from the Dendritic Cell Surface

(A) Antibody uptake as a measure of endocytosis of membrane proteins. If endocytosis of the expressed proteins occurs during exposure of living cells to antibodies in the extracellular medium, some antibodies become trapped in endosomes. These trapped primary antibodies will be accessible to fluorescently labeled secondary antibodies only if membranes are permeabilized after fixation.

(B) VAMP2 is endocytosed from the dendritic cell surface. Sister cultures of neurons transfected with VAMP2-CFP were exposed to antibodies against the extracellular fluorescent protein tag for 20 min at 37°C prior to fixation. Comparison of VAMP2 signal without detergent permeabilization (left panel) and after permeabilization (right panel) revealed a distinct staining pattern in dendrites. The numerous, brightly fluorescent puncta seen in dendrites (arrowheads) only after detergent permeabilization represent endocytosed VAMP2-CFP. Cell surface staining was seen only in the axon (arrows).

(C) Endocytosis of NgCAM from dendrites is undetectable in mature hippocampal neurons. Parallel antibody uptake experiments in cultures transfected with NgCAM revealed no difference between the staining pattern with (right) or without (left) detergent permeabilization. Under both conditions, NgCAM staining was restricted to axons (arrows) and largely absent from soma and dendrites (arrowheads). Scale bar for (B) and (C), 20 μm.

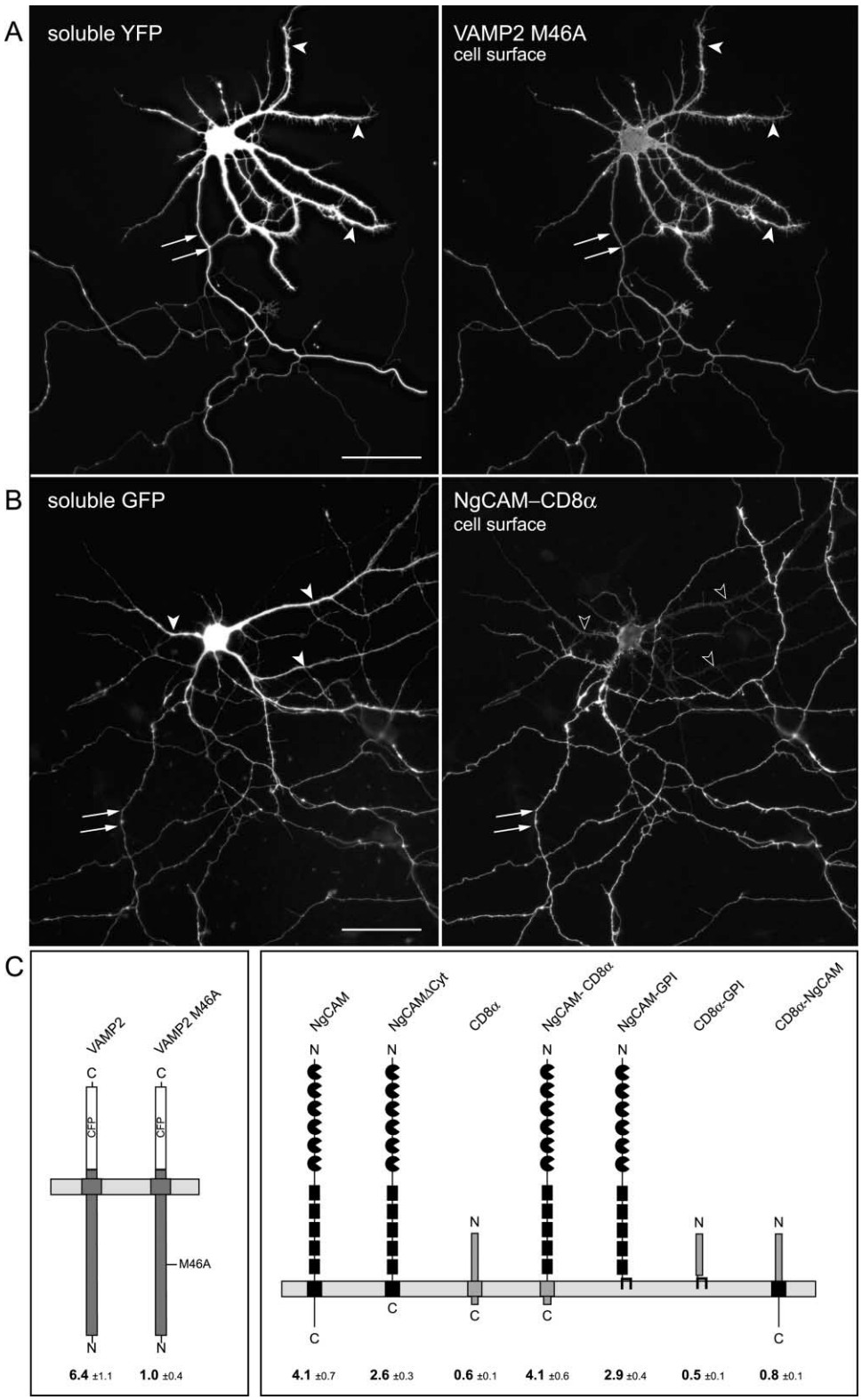


Figure 4. The Cytoplasmic Domain Plays a Critical Role in the Polarization of VAMP2 but Not NgCAM

(A) Mutation of the endocytosis signal in cytoplasmic domain of VAMP2 disrupts its axonal localization. Live immmunolabeling of a neuron expressing YFP (left panel) and CFP-tagged mutant protein VAMP2 M46A (right panel). The mutant VAMP2 was present at similar levels at the cell surface of dendrites (arrowheads) and the axon (arrows). Scale bar, 50 μm.

(B) Polarization of NgCAM does not depend on information in its cytoplasmic domain. Live immunolabeling of a neuron expressing GFP (left

tion of secondary antibodies to unpermeabilized cells). We also visualized VAMP2 that had cycled through the cell surface during a 20 min period, including protein that had been endocytosed (by addition of secondary antibodies to permeabilized cells). The concentration of VAMP2 on the dendritic surface at any instant in time was relatively low, five to ten times less than that on the axonal surface. Despite its low, steady-state level on the dendritic surface, dendrites contained numerous, VAMP2-labeled endosomes. Furthermore, a single amino acid substitution that disrupts VAMP2's endocytosis (Grote and Kelly, 1996) abolished its polarization. Mutant VAMP2 was found at high levels on the dendritic, as well as the axonal surface. The accumulation of endocytosis-deficient VAMP2 on the dendritic surface is unlikely to be due to diffusion from the axonal surface, because a barrier at the axon initial segment prevents the diffusion of axonal plasma membrane proteins into the dendrites (Winckler et al., 1999). These results indicate that VAMP2 is normally delivered to the dendritic membrane but does not accumulate there because it is rapidly endocytosed. Our observations are also consistent with findings on the localization of SNB-1, the *C. elegans* homolog of VAMP2, in a mutant deficient in clathrin-dependent endocytosis (Nonet et al., 1999). A mutation in the UNC-11 gene, which encodes the *C. elegans* homolog of the AP180 adaptor protein (McMahon, 1999), results in the appearance of VAMP2 in dendrites, as well as the axon.

From dendrites, VAMP2 could be targeted to the degradative pathway or transcytosed to the axon, as is the case for pIgR (de Hoop et al., 1995). On the other hand, it is unlikely that VAMP2 reaches the axon exclusively via transcytosis. If transcytosis were the only route for VAMP2 to reach the axon, mutations that blocked its endocytosis would be expected to cause its selective accumulation on the dendritic surface and prevent its appearance in the axonal membrane. Instead, we observed that mutant VAMP2 was present in equal amounts on the surface of axons and dendrites.

In the axon, interactions with specific proteins that are anchored to the submembrane cytoskeleton, such as synapsin or neurexins (Missler et al., 1998; Ferreira and Rapoport, 2002), could act to prevent entry of VAMP2 into coated pits and enhance its retention in the plasma membrane. It is also possible that the higher concentration of VAMP2 on the axonal surface arises because endocytosed VAMP2 is much more efficiently recycled back to the cell surface in axons.

Selective Delivery Is Likely to Account for the Axonal Polarization of NgCAM

Like VAMP2, the cytoplasmic domain of NgCAM contains a well-defined endocytosis motif, and carriers containing NgCAM are transported into both dendrites and axons. Nevertheless, three lines of evidence indicate that endocytosis is not required for the polarization of NgCAM. First, if the NgCAM carriers that are present in dendrites deliver their contents to the dendritic membrane, it should be possible to detect the transient appearance of NgCAM on the dendritic surface and its subsequent endocytosis by exposing living cells to anti-NgCAM antibodies for an extended period of time. We were unable to detect the endocytosis of NgCAM in dendrites in mature neurons. Second, if selective endocytosis mediates the polarization of NgCAM, then mutant constructs that lack the endocytosis signal should be less polarized than the wild-type protein. In fact, such mutant proteins were fully polarized. Third, our analysis revealed that the axonal polarization of NgCAM depends on information contained in the FnIII repeats in its extracellular domain. By analogy with results on apical targeting in epithelial cells (see below), deletions within the ectodomain of NgCAM could well disrupt its sorting, allowing it to enter an inappropriate population of carriers, which deliver cargoes to the dendritic membrane. Mutations in the ectodomain are unlikely to interfere with endocytosis. Indeed, NgCAM mutants lacking the FnIII domains inappropriately reach the dendritic surface, and their endocytosis from the dendritic surface was readily detectable by antibody uptake. These observations strongly imply that wild-type NgCAM is preferentially delivered to the axonal membrane. Additional methods that allow direct visualization of the fusion of NgCAM carriers with the plasma membrane, such as total internal reflection fluorescence microscopy (TIR-FM), will be required to quantify differences in rates of exocytosis of these carriers in axons and dendrites.

If NgCAM is selectively delivered to the axonal plasma membrane while VAMP2 is delivered equally to both axonal and dendritic domains, these two proteins must reach the membrane via different carriers. In addition, the preferential fusion of NgCAM- but not VAMP2-containing carriers with the axonal membrane implies that the former contain unique v-SNAREs (or other proteins that govern interaction with the fusion machinery) and that these proteins interact with axon-specific t-SNAREs or other tethering proteins (Foletti et al., 1999). If this model is correct, it remains to be determined how VAMP2 and NgCAM become segregated into different carriers and where in the cell their trafficking diverges. The latter question could be addressed by expressing NgCAM and VAMP2 tagged with different spectral variants of GFP. This approach would also allow simultaneous visualization of the delivery of these proteins to the membrane by two-color TIR-FM.

panel) and a chimeric protein consisting of the NgCAM ectodomain fused to the transmembrane and cytoplasmic domain of CD8α. The chimeric protein (right panel) was detected predominantly on the axonal surface (arrows), not on dendrites (arrowheads). Scale bar, 50 μm. (C) Quantification of the distribution of constructs to evaluate the role of the cytoplasmic domains of VAMP2 and NgCAM. Axon:dendrite ratios (shown below each construct) were calculated by the method illustrated in Figure 1. Mutation of methionine 46 in VAMP2, which disrupts its endocytosis, also greatly reduced its polarization (axon:dendrite ratio of 1.0 $\pm$ 0.4) compared with wild-type VAMP2 (ratio of 6.4 $\pm$ 1.1). Deletion of the cytoplasmic domain of NgCAM (NgCAMΔCyt) reduced its polarization only slightly (2.6 $\pm$ 0.3). Replacing the transmembrane and cytoplasmic domains of NgCAM with those of CD8α, which lacks an endocytosis signal, or substituting a GPI anchor for the transmembrane and cytoplasmic domains of NgCAM did not affect the axonal polarization of NgCAM. Replacing the NgCAM ectodomain with the ectodomain of CD8α completely abolished axonal targeting (axon:dendrite ratio 0.8 $\pm$ 0.1).

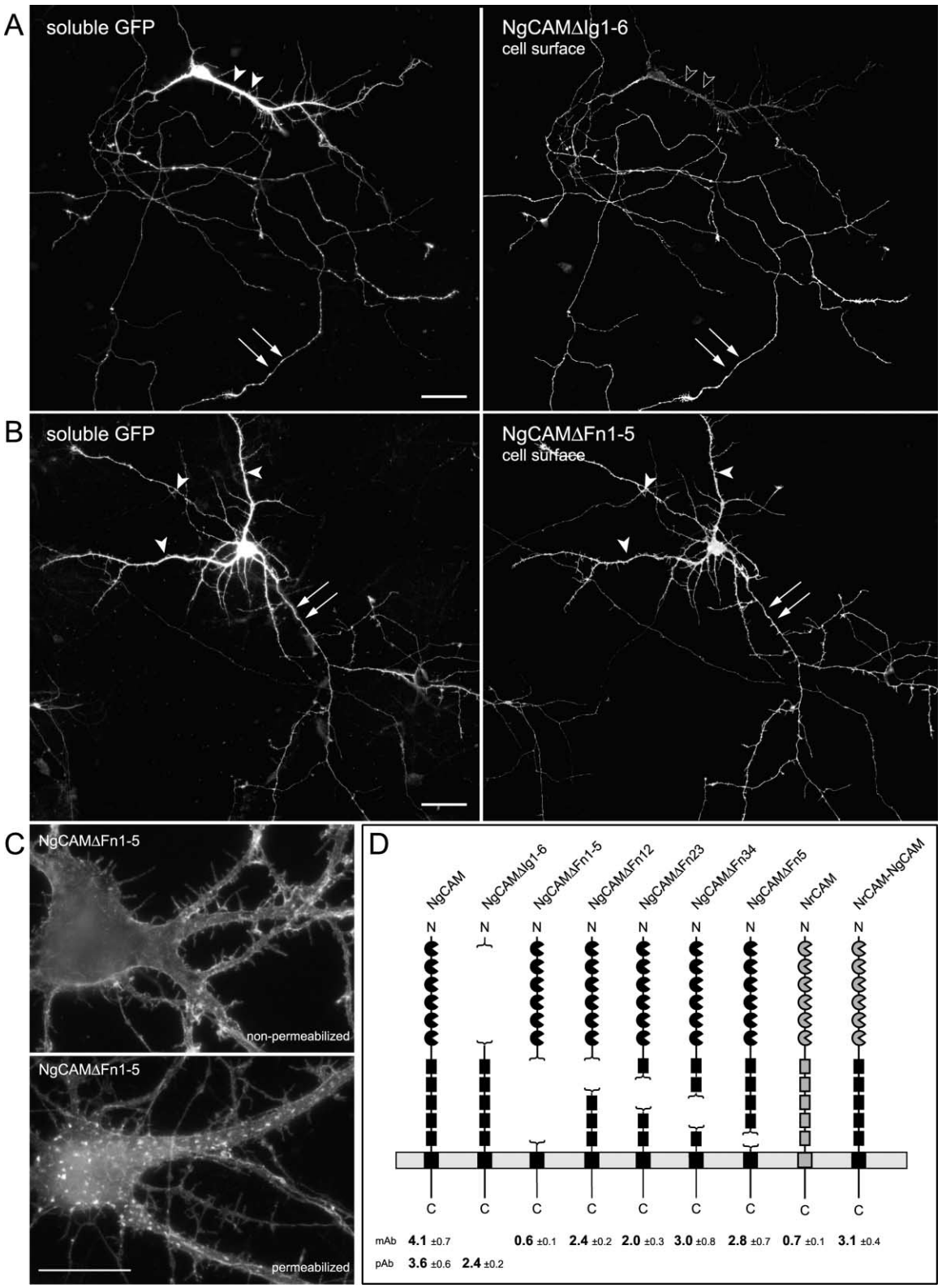


Figure 5. The FnIII Repeats in the Extracellular Domain of NgCAM Are Required for Axonal Polarization

(A) Deletion of the Ig domains does not disrupt the axonal polarization of NgCAM. Live immunolabeling of a neuron coexpressing GFP (left panel) and an NgCAM mutant lacking all Ig-like domains (NgCAMΔIg1-6, right panel) was performed with a polyclonal antibody. Because NgCAMΔIg1-6 showed a reduced cell surface expression, staining was performed 3 days after transfection. The mutant protein was polarized to the axon (arrows) while dendrites were barely labeled (open arrowheads). Scale bar, 50 μm.

(B) Deletion of the FnIII repeats disrupts axonal polarization of NgCAM. This neuron was transfected with GFP (left panel) and an NgCAM

NgCAM Contains an Axonal Targeting Signal in Its Extracellular Domain

Our studies further demonstrate that NgCAM contains an axonal targeting signal that resides within the FnIII repeats in its extracellular domain. Deletion of the fibronectin repeats abolishes the polarization of NgCAM, and addition of the fibronectin repeats to NrCAM, an unpolarized protein, is sufficient to redirect it to the axonal membrane. Interestingly, the signal could not be further dissected by loss-of-function experiments; deleting individual FnIII repeats or pairs of repeats failed to abolish axonal targeting, although the degree of polarization was somewhat reduced. A plausible explanation for this result is that more than one of the FnIII repeats contains information that permits axonal targeting. Whatever features within this region are important for axonal sorting, they appear to be specific to the FnIII repeats in NgCAM. Related proteins such as axonin-1 and NrCAM, which also contain four or five FnIII repeats, are present on the surfaces of both dendrites and axons (Halfter et al., 1994) (Figure 5D).

Many previous studies have addressed the targeting of other axonal (Arnold and Clapham, 1999; El-Husseini et al., 2001; Garrido et al., 2001) and dendritic proteins (West et al., 1997a; Jareb and Banker, 1998; Stowell and Craig, 1999; Lim et al., 2000; Ruberti and Dotti, 2000; Eiraku et al., 2002). In nearly every case, targeting was found to depend on sequences within the proteins' cytoplasmic domains. In contrast, the sorting of NgCAM depends on signals in its ectodomain; the cytoplasmic domain of NgCAM appears to play no role. Interestingly, the targeting of several apical membrane proteins in epithelial cells has also been shown to depend on determinants in their extracellular domains (Rodriguez-Boulan and Gonzalez, 1999; Nelson and Yeaman, 2001). Although there is no apparent homology between these apical targeting signals and sequences within the FnIII domains of NgCAM, it may be significant that all of these signals are located in a region close to the transmembrane domain. By analogy with the role that apical signals play in epithelial protein trafficking (Rodriguez-Boulan and Gonzalez, 1999), we speculate that the FnIII motifs in NgCAM interact with a receptor-like molecule contained within the lumen of the TGN, causing NgCAM to be sorted into axonal transport carriers. Alternatively, NgCAM may be sorted into appropriate carriers by "piggybacking," i.e., its FnIII repeats may bind to the luminal domain of another axonal cargo protein whose cytoplasmic domain interacts with a cytoplasmic sorting receptor (Roos and Kelly, 2000).

Our results also have some bearing on the targeting of proteins that are linked to the membrane via GPI anchors. Dotti and colleagues have argued that the GPI anchor serves to target proteins to the axon (Dotti et al., 1991; Ledesma et al., 1999). Other reports indicate that the presence of a GPI anchor, of itself, is not sufficient to direct axonal targeting (Faivre-Sarrailh and Rougon, 1993; Lowenstein et al., 1994; Kollins et al., 1999). Our results indicate that some GPI-linked proteins are polarized to the axon (NgCAM-GPI), while others are unpolarized (CD8α-GPI), depending on whether or not the polypeptide that composes the ectodomain contains axonal targeting information. Benting et al. (1999) reached a similar conclusion with regard to the role of the GPI linkage in epithelial targeting. While most GPI-linked proteins are found on the apical surface of MDCK cells, sorting signals in the proteinaceous portion of GPI-anchored proteins govern their apical targeting.

What Prevents NgCAM in the Axonal Membrane from Diffusing into the Somatodendritic Domain?

The initial segment, at the origin of the axon, is thought to contain a barrier that prevents axonal membrane proteins from diffusing into the somatodendritic domain (Winckler et al., 1999). In particular, it has been shown that the diffusion of NgCAM within the plasma membrane is much more restricted within the initial segment than in more distal regions of the axon. Winckler and colleagues hypothesize that NgCAM molecules that diffuse into the initial segment from more distal regions of the axon become tethered by binding to submembranous cytoskeletal proteins that are concentrated in the initial segment, including ankyrin G. Although the cytoplasmic domain of NgCAM binds to ankyrin G, our results show that this is not required for maintaining the polarization of NgCAM to the axonal membrane. Deleting the cytoplasmic domain, replacing the cytoplasmic domain of NgCAM with that of CD8α (which lacks an ankyrin binding domain), or replacing the transmembrane and cytoplasmic domains of NgCAM with a GPI anchor did not abolish the polarization of NgCAM. It may be that the restricted diffusion of NgCAM is mediated by interactions between its extracellular domain and the ectodomains of other transmembrane proteins, such as NrCAM or neurofascin, which are tethered at the initial segment (Jenkins and Bennett, 2001). Alternatively, its restricted diffusion may simply reflect steric hindrance due to the high density of transmembrane proteins that are directly tethered to the submembranous cytoskeleton in the initial segment (Hollenbeck and Ruthel, 1999).

mutant lacking all five FnIII repeats (NgCAMΔFn1-5). Strong labeling was detected on the surface of the dendrites (arrowheads) as well as the axon (arrows). Scale bar, 50 μm.

(C) The NgCAM mutant protein that reaches the dendritic surface undergoes endocytosis. Sister cultures transfected with NgCAMΔFn1-5 were exposed to antibodies against the extracellular domain for 20 min at 37°C prior to fixation. The mutant NgCAM protein NgCAMΔFn1-5 was present on the surface of the cell body and the proximal dendrites when analyzed without membrane permeabilization (upper panel). After permeabilization with 0.2% Triton-X100 (lower panel), numerous fluorescent puncta of internalized antibody were present throughout the somatodendritic domain. Scale bar, 20 μm.

(D) Quantification of the distribution of constructs to evaluate the role extracellular domain of NgCAM. Deletion of all six Ig-like domains of NgCAM did not significantly alter its axonal polarization (axon:dendrite ratio of 2.4 $\pm$ 0.2) compared with wild-type NgCAM (3.6 $\pm$ 0.6). In contrast, deletion of the five FnIII repeats reduced the axon:dendrite ratio to 0.6 $\pm$ 0.1, identical to the ratio for the unpolarized protein CD8α. Deletion of single or pairs of FnIII repeats did not significantly change axonal polarization of NgCAM. NrCAM, another member of the L1 family of neural cell adhesion molecules, exhibited an axon:dendrite ratio of 0.7 $\pm$ 0.1, typical of unpolarized proteins. A chimeric protein containing the six Ig-like domains of NrCAM and the five FnIII domains of NgCAM was polarized to the axon (axon:dendrite ratio of 3.1 $\pm$ 0.4).

An Emerging Picture of Neuronal Protein Trafficking

Each step along the neuronal membrane protein trafficking pathway—sorting into carrier vesicles, transport along microtubules, fusion with the plasma membrane, and retention at the cell surface—is a potential "decision site" where molecular selectivity mechanisms could act to govern protein targeting. The picture that is now emerging suggests that the importance of selectivity mechanisms acting at different sites along the trafficking pathways may differ for different classes of polarized proteins. For dendritic proteins, polarization is critically dependent on the selectivity processes that govern sorting (to ensure that the protein enters only the appropriate carrier vesicles) and transport (to ensure that these carriers are prevented from entering the axon) (Burack et al., 2000; Setou et al., 2000; Cheng et al., 2002). This makes sense from the perspective of cellular economics. If dendritic carriers were able to enter the axon, they might travel far from the cell body before eventually returning to reach the dendritic domain. For certain axonal proteins, including NgCAM, polarization appears to depend on the selectivity of sorting and of exocytosis, but not on the selectivity of transport (Burack et al., 2000; Goldstein and Yang, 2000). Carriers containing NgCAM reach both axons and dendrites, but our results indicate that these carriers deliver NgCAM preferentially to the axonal surface. Because dendrites are relatively short, it may not matter if axonal carriers make an excursion to the dendrites en route to the axon. For other axonal proteins, including VAMP2, polarization depends on selective retention, not on selective delivery. Selective retention is governed by events that occur at the plasma membrane, either by binding to adaptors that mediate endocytosis or by binding to anchoring proteins that prevent endocytosis. Targeting by selective retention may be of particular importance for proteins that are restricted to specific microdomains within the axon. For proteins such as sodium or calcium channels, rapid endocytosis from the dendritic membrane coupled with binding to anchoring proteins at nodes of Ranvier or in presynaptic terminals may be sufficient to ensure that the proteins never reach significant concentrations in the dendritic membrane (Garrido et al., 2001; Maximov and Bezprozvanny, 2002). Which of these two mechanisms, selective retention or selective delivery, is the preponderant pathway for polarization of axonal proteins remains to be determined.

Experimental Procedures

Antibodies

NgCAM constructs (kindly provided by P. Sonderegger, University of Zurich) were detected with either a polyclonal goat serum G35 (Fitzli et al., 2000) or culture supernatant of hybridoma clone 8D9 (Developmental Studies Hybridoma Bank, University of Iowa, IA). NrCAM constructs were detected with the polyclonal goat serum G68 (Fitzli et al., 2000). CD8α constructs were detected with a monoclonal antibody DK25 (Dako Corporation, Carpinteria, CA) and VAMP2-CFP constructs with anti-GFP antibody (clones 7.1 and 13.1; Roche Diagnostics, Indianapolis, IN). Biotinylated donkey anti-mouse IgG, biotinylated donkey anti-goat IgG, and Cy3-conjugated donkey anti-goat IgG antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Cy3-conjugated avidin D and FITC-conjugated streptavidin were purchased from Vector Laboratories (Burlingame, CA).

DNA Constructs

The cDNAs encoding chicken NgCAM and NrCAM have been previously described (Buchstaller et al., 1996; Fitzli et al., 2000). The VAMP-CFP construct was obtained from W. Almers (Oregon Health and Science University, Portland, OR). The following constructs were prepared by PCR and subcloned into the expression vector pJPA5 (J. Adelman, Oregon Health and Science University): CD8α-NgCAM (CD8α, aa 1–179; NgCAM, aa 1142–1280), NgCAM-CD8α (NgCAM, aa 1–1141; CD8α aa 182–235), NrCAM-NgCAM (NrCAM, aa 1–630, NgCAM, aa 611–1280), NgCAMΔCyt (aa 1–1173), NgCAMΔIg1-6 (deletion of aa 35–610), NgCAMΔFn1-5 (deletion of aa 609–1141). Chimeric constructs containing a GPI attachment signal were generated by fusing the carboxy-terminal 37 residues of DAF (amplified by PCR) to the ectodomains of NgCAM (aa 1–1141) and CD8α (aa 1–183). The endocytosis-deficient mutant of the VAMP2-CFP (VAMP2-M46A-CFP) was made by PCR-based mutagenesis as described (Grote and Kelly, 1996). The identity of all mutants was confirmed by restriction enzyme analysis and dideoxynucleotide sequencing. Other mutants used in this study have been previously described (Kunz et al., 1998).

Cell Culture and Transfection

Primary neuronal cultures were prepared from hippocampi of E18 rats, as previously described (Goslin et al., 1998). Briefly, dissociated neurons were plated onto poly-L-lysine-treated glass coverslips at a density of 50–100 cells/mm^2 and cocultured over a monolayer of astrocytes. Cells were maintained in Neurobasal medium (LifeTech/GIBO-BRL, Gaithersburg, MD) supplemented with B27 and Glutamax. After 8–11 days in culture, cells were transfected with Effectene (Qiagen, Valencia, CA) using 2–4 μg of plasmid DNA per 6 cm dish culture. Unless otherwise indicated, the distribution of expressed proteins was analyzed by indirect immunofluorescence 20–24 hr after transfection. To assess the role of *N*-glycosylation in NgCAM targeting, tunicamycin (5 μg/ml; Sigma Chemical Co., St. Louis, MO) was added to some cultures 6 hr after transfection, and the cells were processed for immunofluorescence 18 hr later using polyclonal G35 antibody.

Immunofluorescence

Cell surface immunofluorescence was assessed as previously described (Jareb and Banker, 1998). For most experiments, living cells were incubated with primary antibodies for 5 min, rinsed briefly in PBS, and fixed with 4% paraformaldehyde/0.1% glutaraldehyde/0.4% sucrose in PBS for 20 min at 37°C. Cells were then incubated with 0.5 mg/ml $NaBH_4$ in PBS for 15 min to quench autofluorescence generated by glutaraldehyde fixation, extracted with 0.2% Triton X-100 for 5 min at 4°C, and blocked in PBS containing 0.2% fish skin gelatin (Sigma Chemical Co.). Cells were incubated with appropriate biotinylated secondary antibodies for 1 hr at room temperature in the same buffer. After extensive washes, the samples were incubated with Cy3-conjugated avidin D for 45 min. The coverslips were then washed three times with PBS and mounted in Elvanol.

Microscopy and Quantification

Observations were made on a Leica DM-RXA microscope and digital images acquired using a Princeton Instruments Micromax CCD camera (Roper Scientific, Trenton, NJ) controlled by MetaMorph image acquisition and analysis software (Universal Imaging Company, Downingtown, PA). Mature cells with long axons and well-developed dendritic arbors (Developmental Stage 5 of Dotti et al. [1988]) were chosen, based on GFP or YFP labeling. Images illustrating the antibody labeling and the distribution of the soluble marker protein were acquired using a 40× 0.75 N.A. dry objective. Exposure times were adjusted so that the pixel intensities were at least half saturation. The acquired images were corrected for background by subtracting a single image taken with the same settings and no illumination, then corrected for uneven illumination (shading correction) and stored with no further modifications. From these images, montages were created that showed the labeled cell in its entirety, including all axonal branches and the complete dendritic arbor. Using the GFP montage, 1 pixel-wide line segments were traced along all dendrites and representative portions of the axon. Regions where fasciculation occurred were excluded from the analysis. The

lines were then transferred to the images of antibody labeling, the average pixel intensity was measured along each line segment, and these values were imported into Excel software for data analysis. The average background intensity (based on neighboring regions of the culture that contained untransfected cells) was subtracted for both dendrites and axons, and an average axon:dendrite ratio was then calculated for each cell. For each construct, 6–16 cells from at least two different culture preparations were measured. Highly expressing cells were excluded from the quantitative analysis because we noted that such cells often exhibited a less polarized distribution of the transfected construct.

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